THE EVOLUTION OF VIOLACEAE FROM AN ANATOMICAL AND MORPHOLOGICAL PERSPECTIVE[1]

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ABSTRACT

Recent work suggests that *Fusispermum* Cuatrec. and *Rinorea* Aubl. form small clades that are sister to the rest of the Violaceae and that the Goupiaceae is sister to the Violaceae. However, little is known about the morphology and anatomy of these phylogenetically critical groups. In this paper I present aspects of the morphology and anatomy of stem, node, leaf, flower, and seed of three species of *Fusispermum* and seven species of *Rinorea*, as well as *Goupia glabra* Aubl. (Goupiaceae), which is the outgroup. Placing this variation in the context of hypothesized phylogenetic relationships, I found *Fusispermum* to have unique pentalacunar nodes, heterogeneous pith, and elongated seeds, while *Goupia* Aubl. has a unique 5-carpellate gynoecium with marginal styles and a tegmen with U-shaped thickenings. Furthermore, variation in androecium and nectary links the distinctive androecium so common in the Violaceae with more conventional structures found in other taxa of the parietal placentation group of Malpighiales. Strengthening our basic knowledge of anatomy and morphology in these groups is an essential prerequisite for understanding the evolution and diversification not only of Violaceae but of Malpighiales as a whole.

Key words: Anatomy, flower, *Fusispermum*, *Goupia*, Goupiaceae, leaf and seed morphology, Malpighiales, *Rinorea*, stem, Violaceae.

The violet family (Violaceae) is predominantly tropical in distribution and contains 23 genera and more than 900 species (Feng, 2005; Tokuoka, 2008). The family is supported as monophyletic (Feng, 2005; Tokuoka, 2008), although phylogenetic relationships within it are still unclear (Feng, 2005; Tokuoka, 2008). The Violaceae encompasses a great diversity of vegetative and floral variation. Habits may vary from herbs to lianas to trees, with flowers ranging from zygomorphic (monosymmetric) and strongly spurred to actinomorphic (polysymmetric) and unspurred. Anther variation is considerable, and there is variation in shape and structure of the androecium in the Neotropical *Rinorea* Aubl., especially in the connective scales. The fruits range from an indehiscent nut or fleshy berry to loculicidal capsules, sometimes explosively dehiscent; the seeds are small to noticeably large and may be winged or carunculate (Hekking, 1988).

Recent studies (Wahlert et al., 2014) have changed our understanding of the phylogeny of Violaceae by elucidating unsuspected or lesser-known groups that are prime candidates for the study of morphology and anatomy. Within the Violaceae, clades for *Fusispermum* Cuatrec. and *Rinorea apiculata* Hekking (Wahlert & Ballard, 2012) are strongly supported as being successive sisters to the rest of the family (Fig. 1). The uncommon *Fusispermum* is a Neotropical genus with three species, ranging from Costa Rica to Peru. In contrast, *Rinorea* is a common pantropical genus with 49 species in the Neotropics (Wahlert & Ballard, 2012).

Another development that affects our understanding of the Violaceae is their phylogenetic association with the Goupiaceae, which contains *Goupia* Aubl. with two species, one from Central America (*G. guatemalensis* Lundell), and the other (*G. glabra* Aubl.) from northern South America to the north of the Amazon basin. The phylogenetic position of the Goupiaceae has long been poorly understood. *Goupia* was included in the Celastraceae by Bentham and Hooker (1862) as the subfamily Goupioideae, a view later accepted by Cronquist (1981). The genus has been recognized as its own family (e.g., Simmons et al., 2001). However, *Goupia* has not previously been associated with the Violaceae or with any other family

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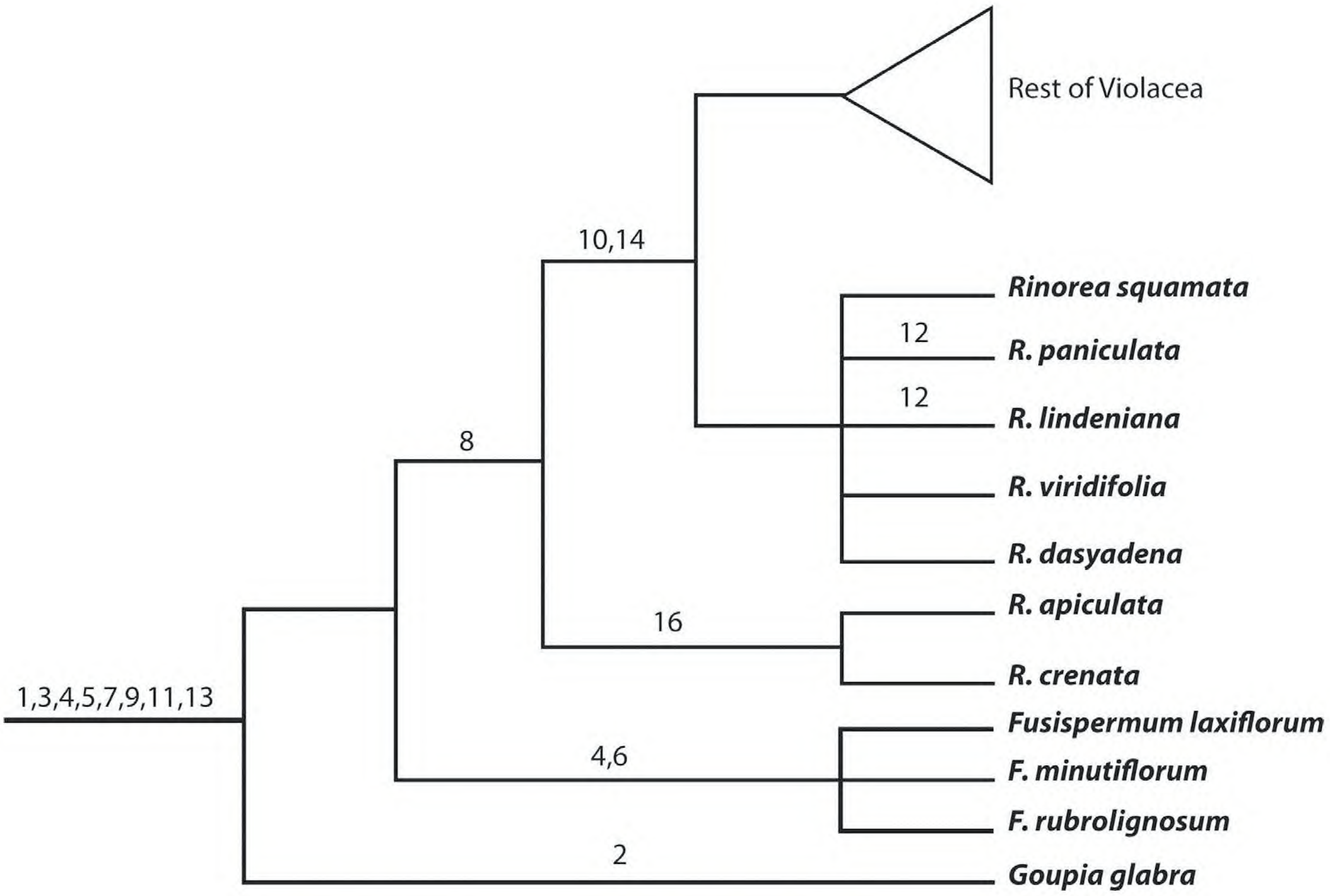


Figure 1. Summary tree diagram for relationships between *Fusispermum* Cuatrec. and *Rinorea* Aubl. (Violaceae) and the outgroup exemplar *Goupia glabra* Aubl. (Goupiaceae). Morphological and anatomical character states useful for understanding relationships among these taxa are mapped on the tree. Those character states found at the stem of this tree (1, 3, 4, 5, 7, 9, 11, 13, 15; see Table 2) may be found in other members of the parietal placenta clade. Mapped characters include (1) cylindrical stem, (2) fluted stem, (3) homogeneous pith, (4) heterogeneous pith, (5) trilacunar nodal anatomy, (6) pentalacunar nodal anatomy, (7) nectary disc, (8) gland adnate to filament, (9) connective scale apical, (10) connective scale basal, (11) style straight, (12) style curved, (13) seed with exotegmen with sclereids (one layer), (14) seed with exotegmen with fibers (four to six layers), (15) stigma area not expanded, and (16) stigma area flared outward.

in the parietal placentation group within the Malpighiales. Placentation in the Goupiaceae has been distinguished as basal-axile (Stevens, 2012).

Simmons et al. (2001), in their study of the relationships of the Celastraceae, first suggested that its subfamily Goupioideae belonged to the Malpighiales. Feng (2005) and Wurdack and Davis (2009) placed it, as the Goupiaceae, as sister to the Violaceae, and both analyses find strong support for this position. The Goupiaceae are part of a clade that includes the Achariaceae, Lacistemataceae, Passifloraceae, and Salicaceae. *Goupia* remains the only taxon in this group that has axile placentation, with all the others presenting parietal placentation (Wurdack & Davis, 2009).

Few comprehensive studies have been published on the Violaceae, and the morphology of the family, particularly *Fusispermum* species and the *Rinorea apiculata* clade, is largely unknown (Wahlert & Ballard, 2012). Even less is known about floral evolution and development (but cf. Arnal, 1945; Feng, 2005). Most studies have focused on *Viola* L., a genus widely distributed across both hemispheres. But *Viola* is atypical in the family in being largely herbaceous, with derived character states, and of largely temperate distribution (Metcalfe & Chalk, 1972; Corner, 1976; Feng, 2005). In contrast, the majority of the family are woody and tropical taxa, so extrapolation from the derived states in *Viola* to the other genera in the family may be inappropriate or misleading.

Metcalfe and Chalk (1972) summarized what little is known of the vegetative anatomy of *Goupia*, *Fusispermum*, and *Rinorea*. For seeds, Hekking (1984) briefly described the external morphology of the seeds of *Fusispermum*. Corner (1976) investigated the seed anatomy of *Viola*, *Hybanthus* Jacq., and two species of Malesian *Rinorea*. The broadest survey of seed anatomy of Violaceae is by Plisko (1992), but only 14 species were studied there. The seed anatomy of *G. glabra* was described by Melikian and Sarinov (2000; but see below). The seed anatomy of *Fusispermum* and the *R. apiculata* group has not been previously described.

In an attempt to develop a better understanding of the evolutionary history of Violaceae, I examine here the stem, leaf, flower, and seed of three species of *Fusispermum*, of the *Rinorea apiculata* clade (*R. apiculata* and *R. crenata* S. F. Blake), and *Rinorea* s. str. (five species considered), as well the outgroup exemplar *Goupia glabra* (Goupiaceae). For these taxa, I describe anatomical and morphological characters of the stem, node, leaf, androecium, gynoecium, and seed anatomy and morphology using scanning electron microscopy (SEM) and light microscopy (LM). I then place this information in the context of proposed phylogenetic relationships among malpighiacous taxa in the Violaceae and Goupiaceae.

Materials and Methods

During the summer of 2010 several field trips were made to Central and South America to obtain plant material. In Bolivia, collection and observations were made in Departamento de la Paz, Provincia Franz Tamayo, Parque Nacional Madidi, Laguna Chalalan, 14°25′39.49″S, 67°54′57.68″W, at an altitude of 350 m. In Colombia, our collection site was Antioquia, Municipio de San Luis, Cañon de Río Claro, Reserva Natural, 05°53′N 074°39′W, 350 m. In Costa Rica, exemplars were taken from Puntarenas, Osa, Sierpe, Reserva Forestal Golfo Dulce, Estación Biológica Los Charcos de Osa, 08°40′18″N 83°30′17″W, 70 m. In Peru, field work occurred in Distrito de Loreto, Provincia de Maynas, carretera Bella Vista–Mazán, 3°31′41.9S, 73°05′45.8W, 86 m. Voucher specimens (cf. Table 1) of the newly collected material were deposited in Costa Rica (INB), Bolivia (LPB), Peru (USM), Colombia (COL, HUA, JAUM), and in the United States (MO). When additional material was needed, I used collections from the Missouri Botanical Garden (MO). No material of *Rinorea oraria* Steyerm. & A. Fernández, which is the third known member of the *R. apiculata* group, was available.

In the field, stem sections, leaves, flowers, and fruits were preserved in 70% ethanol. Leaves from herbarium specimens were used to complete the taxonomic sampling (Table 1). Dried leaves were rehydrated by boiling in water with a drop of detergent before examination. Cross sections of leaves and petioles were handmade, using a razor blade. Microtome sections of seeds were also made. Cresyl violet acetate (CVA) was used for tissue staining, and a 20% calcium chloride solution ($CaCl_2$; Ogburn & Edwards, 2009) was used as a mounting solution (Keating, 1996, 2000). A Canon Power Shot A640 camera (Canon, Tokyo, Japan) coupled to an Olympus BX40 microscope (Olympus, Tokyo, Japan) was used to record all light microscopic images. For SEM imagery, plant material was dehydrated in an increasing ethanol series and then critical-point dried (using an EMITech-K 850 critical-point dryer [EMITech Inc., Fall River, Massachusetts, U.S.A.]). The samples were affixed to supports using carbon adhesive tape and were gold-coated (using a Denton vacuum sputter coater [Denton, Moorestown, New Jersey, U.S.A.]) and were examined using a JEOL Neoscope JCM 5000 SEM (JEOL, Tokyo, Japan). The SEM was used to study cuticle and stomatal microdetail, as well as pollen, fruit, and flower ultrastructure.

Results

YOUNG STEMS

All species studied had lenticels on the stem and a cork cambium that developed early and was superficial in position. A band of collenchyma tissue ca. five to 10 cells broad was observed in the outer cortex. *Goupia glabra* (Goupiaceae) lacked crystals and druses in the collenchyma; however, all Violaceae studied had rhombic crystals and druses of calcium oxalate there. No sclereids or other idioblastic cells were observed in the stem cortex. The stem vasculature of all species studied was eustelic. The central vascular cylinder was noted as more or less circular in transverse section, except in *G. glabra*, where the vascular cylinder was fluted (Fig. 2A). The outermost phloem was completely surrounded by a band of pericyclic fibers. Stem pith was well developed in all species and was usually made up of small, unlignified cells that were more or less similar in size. In *G. glabra*, pith cells were ca. 20–50 μm in diameter (Fig. 2A); in all *Rinorea* species studied, pith cells were 15–40 μm across; in all species the walls of the pith cells were fairly thin, ca. 4–5 μm across (Fig. 2B, C, *R. crenata, R. paniculata* (Mart.) Kuntze, respectively). In contrast, in *Fusispermum laxiflorum* Hekking (Fig. 2D), *F. minutiflorum* Cuatrec., and *F. rubrolignosum* Cuatrec., the pith cells were larger and variable in size, 50–200 μm in diameter; the cell walls were thinner, ca. 2–3 μm across. Pith in *Fusispermum* can be considered heterogeneous in contrast to the more homogeneous condition confirmed in both *Goupia* and *Rinorea* exemplars.

STEM NODES

Goupia glabra (Fig. 2E) and *Rinorea crenata* (Fig. 2F), as did all *Rinorea* exemplars, had three-trace, trilacunar nodes. However, *Fusispermum laxiflorum*

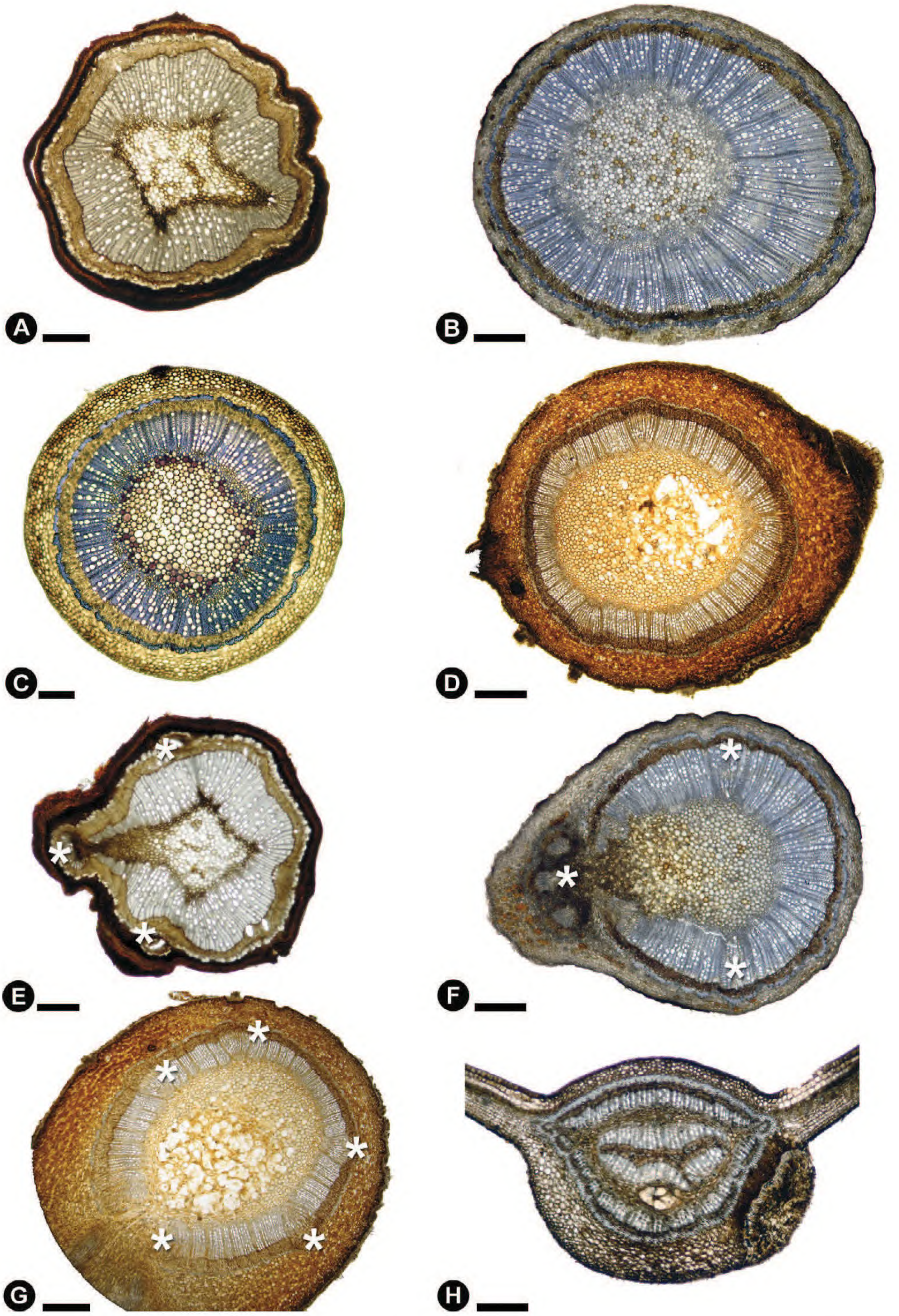


Figure 2. Light microscopic images of stem and leaf blade sections among representative *Rinorea* Aubl. and *Fusispermum* Cuatrec. (Violaceae) and *Goupia* Aubl. (Goupiaceae, outgroup). —A. Transverse stem section, *G. glabra* Aubl. (*Thomas 3992*). —B. Transverse stem section, *R. crenata* S. F. Blake (*S. E. Hoyos-Gómez 1115*). —C. Transverse stem section, *R. paniculata* (Mart.) Kuntze (*S. E. Hoyos-Gómez 1110*). —D. Transverse stem section, *F. laxiflorum* Hekking (*S. E. Hoyos-Gómez 1119*). —E. Internodal stem anatomy, *G. glabra* (*Thomas 3992*). —F. Internodal stem anatomy, *R. crenata* (*S. E. Hoyos-Gómez 1115*). —G. Internodal stem anatomy, *F. laxiflorum* (*S. E. Hoyos-Gómez 1119*). —H. Leaf blade midrib, *F. laxiflorum* (*S. E. Hoyos-Gómez*

(Fig. 2G), *F. minutiflorum*, and *F. rubrolignosum* had pentalacunar nodes, with the vascular traces widely spaced from the central cylinder. In all the species the stipules were vascularized from the lateral traces.

LEAVES

In transverse sections taken at the midpoint of the petiole, peripheral collenchyma tissue was found in all species exemplars. The cells were tanniniferous and contained rhombic crystals and druses, except in *Goupia glabra* (Fig. 3A), which lacked rhombic crystals but did have druses. In all sampled species except *Rinorea apiculata* (Fig. 3B), the vascular tissue formed a more or less closed and somewhat dorsiventrally flattened cylinder with phloem on the outside, xylem on the inside, and the vascular cylinder surrounded by fibers. In *R. apiculata* the main petiole bundle was arcuate, and even there the edges of the arc were strongly incurved. The petiole pith was usually unlignified, but there were dispersed fibers in the pith of the petioles in *G. glabra* (Fig. 3A). All the examined species in *Fusispermum* and *Rinorea* had druses in the pith cells, but *G. glabra* did not. As was seen in the stem cross section, *F. laxiflorum* had much larger and thinner-walled pith cells (Fig. 3C, D) than those of the other examined species.

There were rib traces on either side of the main petiole bundle, usually in adaxial to lateral positions; these traces consisted of arcuate bands of vascular tissue accompanied by a few fibers, especially adjacent to the phloem. There were four rib traces, two on each side of the main bundle, typically seen in *Rinorea apiculata* (Fig. 3B), *R. crenata*, and *R. paniculata*. However, in *R. viridifolia* Rusby, *R. dasyadena* A. Robyns, and *R. squamata* S. F. Blake, only two rib traces were present, one on each side, while in all four specimens of *R. lindeniana* (Tul.) Kuntze (Fig. 3E) examined there were three adaxial rib traces, two on one side and one on the other side above the vascular cylinder.

Fusispermum laxiflorum (Fig. 3C, D), *F. minutiflorum*, and *F. rubrolignosum* have a rather complex petiole vasculature. At the very base of the petiole, immediately after traces depart to the stipules, the vasculature consists of a larger central vascular cylinder and two lateral cylinders on either side, the latter representing the lateral traces. The lateral and central bundles merge and reorganize, becoming broadly incurved and arcuate with a pair of adaxial vascular strands. Finally, toward the apex of the petiole leading into the blade, the vasculature became a more or less complete cylinder surrounding a smaller elliptical cylinder of xylem with phloem on the inside; in addition, there are two small lateral wing bundles on either side of the central cylinder.

In all the *Fusispermum* and *Rinorea* species studied, the leaves were dorsiventrally oriented, with scattered uniseriate hairs on both sides but principally on the abaxial side; *Goupia glabra* had scarce uniseriate hairs on the abaxial side only. Leaf blades varied in thickness from the thickest, ca. 270 μm, in *F. laxiflorum* (Fig. 3F), to ca. 190 μm in *G. glabra* and ca. 150–180 μm among the seven sampled species of *Rinorea* (Fig. 3G, H, *R. apiculata* and *R. squamata*, respectively). In both *G. glabra* and *F. laxiflorum* (Fig. 3F) there was well-defined palisade tissue: with two palisade layers seen in the former and two to three layers in the latter. However, in the seven *Rinorea* species sampled, the palisade tissue was irregularly organized as two to four layers and was not strongly differentiated from the rest of the mesophyll (Fig. 3G, H, *R. apiculata* and *R. squamata*, respectively). No sclereids or other idioblastic cells were observed in either *Fusispermum* or *Rinorea* exemplars. There was some chlorophyllous tissue on the adaxial side of the midrib among the seven species of *Rinorea* but not in *F. laxiflorum* or *G. glabra*. Otherwise the midrib was transcurrent, and smaller veins were embedded.

The vascular tissue in the midrib of the 11 species studied consisted of two arcuate bands of fibers surrounding the vascular tissue. In *Goupia glabra* and *Fusispermum laxiflorum* alone there were also fibers in the medullary tissue of the bundle, and in *F. laxiflorum* they were made up of thin-walled cells like those of the stem pith. In *F. laxiflorum* there were two additional bands of vascular tissue in the midrib that were continuous with the elliptical central cylinder in the petiole.

All 11 species studied had hypostomatic leaves and anomocytic stomata, the stomata being ca. 16–20 μm long. In the seven species of *Rinorea* there were usually three epidermal cells that were directly adjacent to the guard cells, although these adjacent cells were not otherwise distinguishable from the surrounding epidermis. This differed in other taxa in that *Goupia glabra* had four to five epidermal cells adjacent to the stomata, and in *Fusispermum laxiflorum* there were three to five cells directly abutting the stomata.

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1119). Scale bars in A, D, E, G = 500 μm; scale bars in B, C, F = 350 μm; scale bar in H = 700 μm. Asterisks indicate nodes in E–G. All collections are at MO.

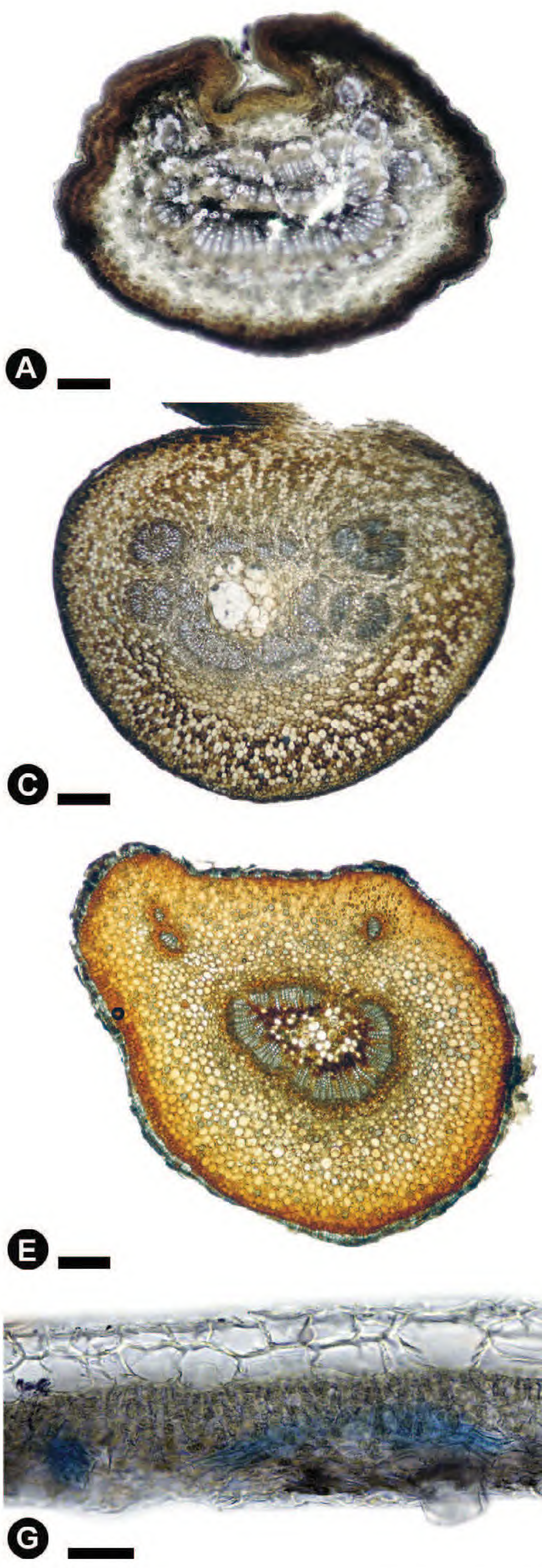


Figure 3. Light microscopic images of leaf petiole and blade sections among representative *Rinorea* Aubl. and *Fusispermum* Cuatrec. (Violaceae) and *Goupia* Aubl. (Goupiaceae, outgroup). —A. Transverse petiole section, *G. glabra* Aubl. (*Thomas 3992*). —B. Transverse petiole section, *R. apiculata* Hekking (*F. Hurtado 2964*). —C, D. Transverse petiole section, *F. laxiflorum* Hekking (*S. E. Hoyos-Gómez 1119*). —E. Hand section of leaf blade, *R. lindeniana* (Tul.) Kuntze (*S. E. Hoyos-Gómez 1135*). —F. Hand section of leaf blade, *F. laxiflorum* (*S. E. Hoyos-Gómez 1119*). —G. Hand section of leaf blade, *R. apiculata* (*P. Nuñez 6026*). —H. Hand section of leaf blade, *R. squamata* S. F. Blake (*S. E. Hoyos-Gómez 1114*). Scale bars in A = 150 μm; B, E = 200 μm; C, D = 300 μm; F–H = 50 μm. All collections are at MO.

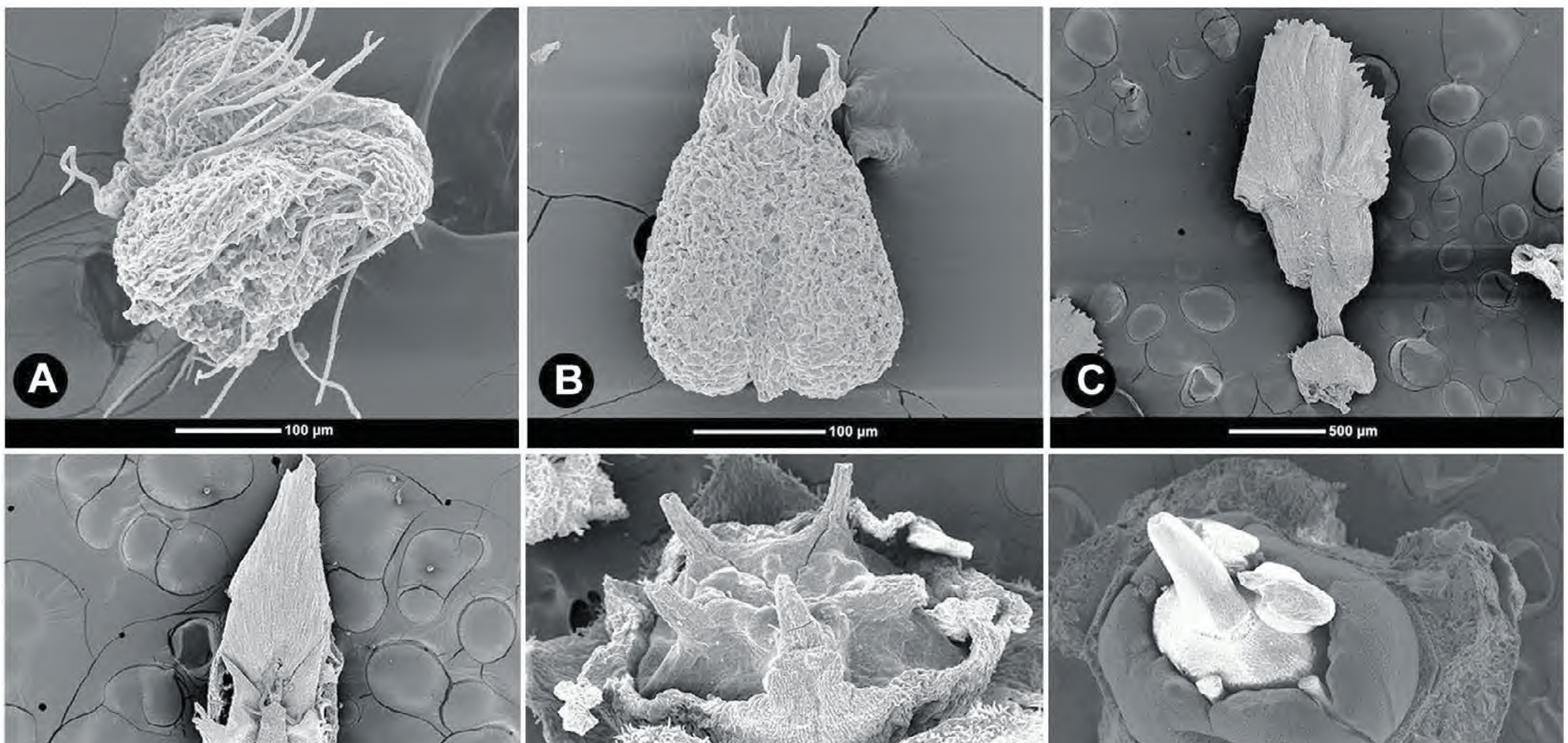


Figure 4. SEMs of androecia and gynoecia among representative *Rinorea* Aubl. and *Fusispermum* Cuatrec. (Violaceae) and *Goupia* Aubl. (Goupiaceae, outgroup). —A. Androecium, *G. glabra* Aubl. (*B. E. Hammel 21278*). —B. Androecium, *F. laxiflorum* Hekking (*G. S. Hartshorn 2929*). —C. Androecium, *R. crenata* S. F. Blake (*M. H. Grayum 10069*). —D. Androecium, *R. lindeniana* (Tul.) Kuntze (*S. E. Hoyos-Gómez 999*). —E. Gynoecium, *G. glabra* (*B. E. Hammel 21278*). —F. Gynoecium, *F. laxiflorum* (*Hartshorn 2929*). —G. Gynoecium, *R. crenata* (*M. H. Grayum 10069*). —H. Gynoecium, *R. viridifolia* Rusby (*S. E. Hoyos-Gómez 1003*). —I. Gynoecium, *R. lindeniana* (*S. E. Hoyos-Gómez 1127*). Scale bars in A, B = 100 µm; C, F, I = 500 µm; D, E, G = 200 µm; H = 1000 µm. All collections are at MO.

ANDROECIA

In all 11 species studied, stamen filaments were glabrous. In *Goupia glabra* these filaments were ca. 200 µm long, and the anther thecae were ca. 260 µm long; in *Fusispermum laxiflorum* they were ca. 500 µm and 240 µm long, respectively. In the seven *Rinorea* species examined the filaments ranged from ca. 300 µm to 950 µm long, and the anther thecae were comparatively longer than in *G. glabra* and *F. laxiflorum*, ranging from ca. 530 µm to 1100 µm long. *Goupia glabra* was the only species to have trapezoid anther shapes (Fig. 4A); the remaining 10 species exemplars had ellipsoid to ovoid anthers. In all species dehiscence was introrse via longitudinal slits in each theca.

In *Goupia glabra*, the androecial connective formed a thick, blunt prolongation beyond the thecae ca. 90 µm long, with cylindrical hairs ca. 230 µm long that were scattered on both the connective and thecae (Fig. 4A). In *Fusispermum laxiflorum* (Fig. 4B), *F. minutiflorum*, and *F. rubrolignosum*, the prolongation of the connective was less massive, ca. 80 µm long, with an irregular fringe apically, while in *Rinorea apiculata* and *R. crenata* (Fig. 4C) the connective formed a broad apical prolongation that exceeded the thecae in length, ca. 930–1100 µm long, with a fimbriate margin. In the remaining five *Rinorea* species sampled, the thecae were not evident from the dorsal side; the connective formed a slightly fringed acute to triangular projection ca. 1300–2000 µm long. Only in *R. apiculata* and *R. crenata* (Fig. 4C) were there flattened hairs on both the adaxial and abaxial surfaces of the anther thecae; in *R. squamata*

there were scattered cylindrical hairs adaxially on the thecae. The remaining Violaceae exemplars had glabrous anthers.

In *Goupia glabra* and *Fusispermum laxiflorum*, *F. minutiflorum*, and *F. rubrolignosum*, an apparently warty disc-like structure with epidermal stomata surrounded the stamens but was quite separate from the anthers. In *G. glabra* this lobed disc completely surrounded the flower (Fig. 4E), with the lobes alternating with the staminal radii. In *F. laxiflorum* (Fig. 4F), *F. minutiflorum*, and *F. rubrolignosum*, the massive disc consists of five lobes alternating with the stamens.

By contrast, the seven examined species of *Rinorea* lacked a disc but did have an abaxial gland-like structure at the base of the filaments; this has hairs in *R. lindeniana* (Fig. 4D), *R. viridifolia*, and *R. paniculata*. In both *R. apiculata* and *R. crenata* (Fig. 4C), this gland was massive, ca. 190 μm long, hemispherical, and joined at the very base with adjacent glands. In the other five species of *Rinorea*, glands were distinct from one another and borne on the filament. In the 11 species studied, there were stomata on the disc and glands consistent with their being nectariferous.

GYNOECIA

Goupia glabra has a glabrous gynoecium of five carpels with five styles borne toward the margin of the carpels (Fig. 4E). Each style, only ca. 200 μm long, has an adaxial furrow and tapers somewhat toward a perhaps hollow but otherwise undistinguished apex. Placentation is axile with ca. five to six ovules per carpel.

All 10 species exemplars of the Violaceae had three fused carpels with parietal placentation. There were many ovules per carpel in *Fusispermum laxiflorum*, *F. minutiflorum*, and *F. rubrolignosum*, but only one ovule per carpel in the seven species of *Rinorea* examined. In *F. laxiflorum* (Fig. 4F), *F. minutiflorum*, *F. rubrolignosum*, *R. apiculata*, and *R. crenata*, the ovary was glabrous, but in other species such as *R. viridifolia* (Fig. 4H), the ovary was pubescent. Styles of all exemplars were continuous with the ovary. In *F. laxiflorum* (Fig. 4F), *F. minutiflorum*, and *F. rubrolignosum* the straight style was narrowed distally to the apex, which had an inconspicuous depression, while in *R. apiculata* and *R. crenata* (Fig. 4G) the style was conspicuously flared at the apex. *Rinorea viridifolia* and *R. squamata* had straight styles and stigmas rather like those of *F. laxiflorum* (Fig. 4F). *Rinorea lindeniana* and *R. paniculata* had sigmoid styles, subapically swollen in *R. lindeniana*, but in both species the stigmatic apex resembled that of the other species of *Rinorea* examined.

SEEDS

The outgroup representative *Goupia glabra* had dark brown ovate seeds ca. 2–3 mm long (Fig. 5A), with the anticlinal walls of the exotesta forming a reticulum (Fig. 5B, C). In transverse section the exotesta was two, perhaps three, cell layers thick and ca. 60–80 μm across; the outer periclinal walls of the exotesta were slightly thickened. There was also a single layer of exotegmic sclereids, these cells being ca. 60 × 100 μm and massively thickened on the anticlinal and inner periclinal walls and also pitted (Fig. 5B, C). *Goupia glabra* lacked crystals in the seed coat. Its straight embryo occupied half the length of the seed and was surrounded by copious oily endosperm.

In contrast, *Fusispermum minutiflorum* (Fig. 5D) and *F. rubrolignosum* had pale brown elongated seeds, ca. 2–3 mm long. The seed had what appears to be a wing down one side where the seed coat was much thicker and was ca. 11 to 16 cells across. There was also a single layer of exotegmic sclereids, the cells being ca. 100 × 10 × 15 μm, and the cell walls being massively lignified all around and pitted (Fig. 5E). There were druses in the testa, as were seen in all seven *Rinorea* species studied. The straight embryo occupied 3/5 the length of the seed, and an oily endosperm surrounded the embryo (Fig. 5D).

Rinorea apiculata and *R. crenata* (Fig. 5G) have light brown ovoid seeds, ca. 4–5 mm long. In transverse section, the testa consisted of unlignified, more or less isodiametric cells three to four layers thick. There was a single layer of exotegmic cells ca. 150 × 10 × 70 μm (Fig. 5F, *R. apiculata*). These cells had more or less pitted walls that were all equally thickened. Only in *R. apiculata* and *R. crenata* among the seven species studied here were there rhombic crystals in the testa. The straight embryo occupied four fifths of the length of the seed and was surrounded by copious oily endosperm surrounding the embryo.

Rinorea squamata, *R. viridifolia* (Fig. 5H), and *R. dasyadena* had brown globose seeds ca. 6–9 mm across. The testa consisted of eight to 10 layers of unlignified flattened cells, with individual cells isodiametric and ca. 20 μm across (Fig. 5H, *R. viridifolia*). There is a middle layer of lignified cells about four to six cells across, the cells being ca. 190 × 15 × 30 μm (Fig. 5H). The cell walls were equally thickened and more or less consistently pitted. The embryo occupied four fifths of the length of the seed,

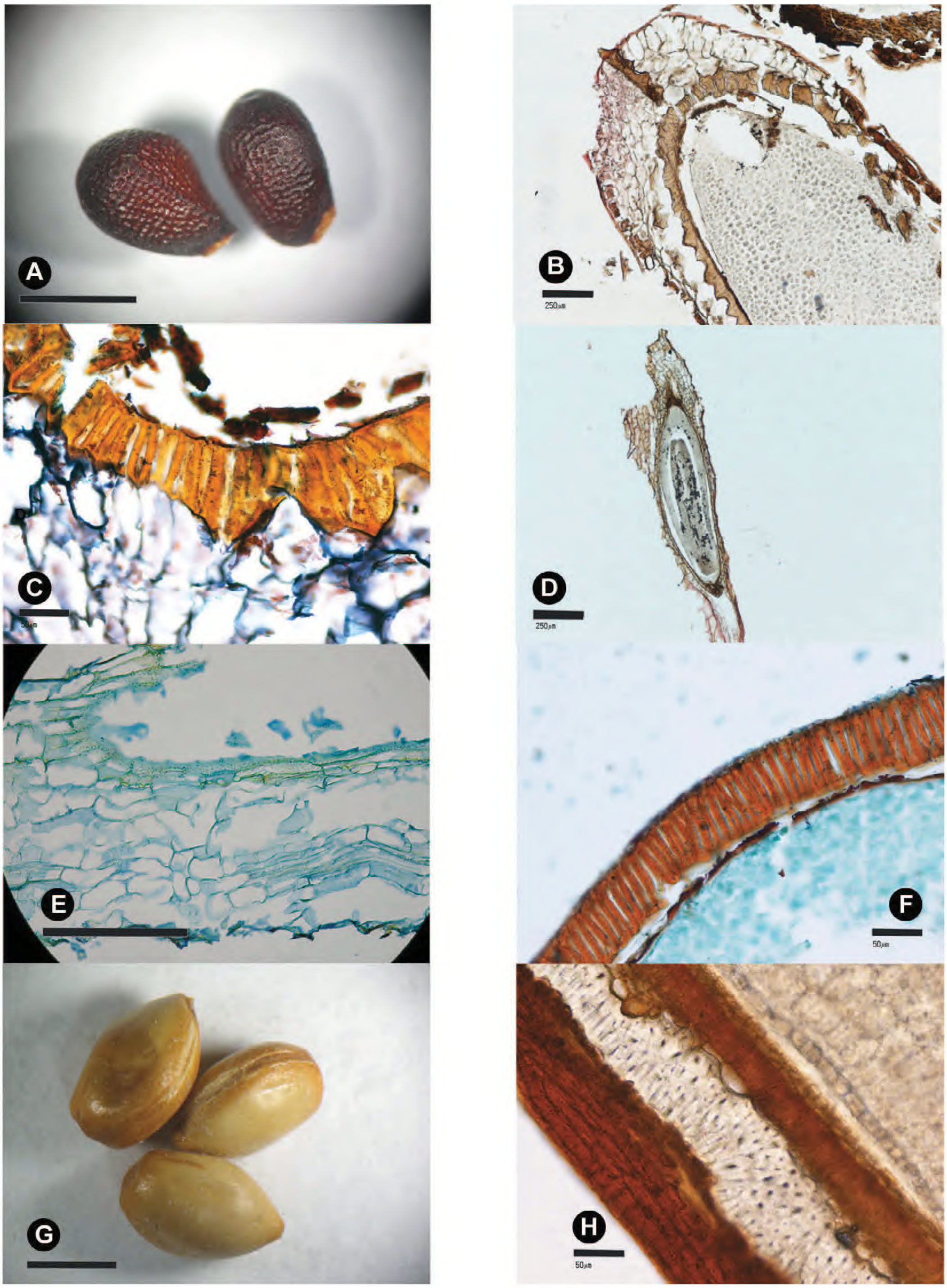


Figure 5. Light microscopic images of seeds and transverse sections among *Fusispermum* Cuatrec. and *Rinorea* Aubl. (Violaceae) and *Goupia glabra* Aubl. (Goupiaceae). —A. Two intact seeds, *G. glabra*. —B. Transverse seed section, *G. glabra*. —C. Transverse seed section, *G. glabra*. (A–C taken from *T. W. Henkel 3245*.) —D, E. Transverse seed sections, *F. minutiflorum* Cuatrec. (*J. Espina 1330*). —F. Transverse seed section, *R. apiculata* Hekking (*C. E. Cerón 5478*). —G. Three intact seeds, *R. crenata* S. F. Blake (*B. E. Hammel 18113*). —H. Transverse seed section, *R. viridifolia* Rusby (*S. E. Hoyos-Gómez 1004*). Scale bars in A = 3000 µm; B–E = 250 µm; F, H = 50 µm; G = 2000 µm. All collections are at MO.

and the cotyledons had several sinuous foldings; copious oily endosperm surrounded the embryo.

Discussion

The results of this study suggest a number of morphological and anatomical characters that are useful for understanding the Neotropical relationships among the Goupiaceae (i.e., *Goupia glabra*) and the two basalmost genera among the Violaceae (i.e., *Fusispermum laxiflorum*, *F. minutiflorum*, and *F. rubrolignosum*; *Rinorea apiculata*, *R. crenata*, *R. lindeniana*, *R. squamata*, *R. viridifolia*, *R. dasyadena*, and *R. paniculata*). Several significant character states (16) are mapped on a summary tree diagram for phylogenetic relationships among these Violaceae and Goupiaceae (Fig. 1). Observations are confirmed here, often for the first time, and are integrated with the sparse literature available (cf. Metcalfe & Chalk, 1972, anatomy; Corner, 1976, seeds).

Overall, these results show the value of anatomical studies when related to a phylogeny. Nodal anatomy is a good example. *Goupia glabra* was considered to belong to Celastraceae by some authors based on gross morphology (Cronquist, 1981). However, the nodes of Celastraceae are overwhelmingly unilacunar (Sinnott, 1914; Cronquist, 1981). The three-trace, three-gap nodes of *G. glabra* are more consistent with its new position, more so since other Celastraceae with other than unilacunar nodes (e.g., *Perrottetia* Kunth, *Bhesa* Buch.-Ham. ex Arn.) have also been removed from Celastraceae to clades where these nodes are common (Stevens, 2012). Furthermore, the pentalacunar nodes of *Fusispermum laxiflorum* are uncommon in Malpighiales and are a potential apomorphy for the genus, and it can be noted here that nodal anatomy is associated with a distinctive petiole anatomy.

The fluted stem pith of *Goupia glabra* is an apparently trivial difference, which is nevertheless consistent within the Goupiaceae and contrasts with the cylindrical pith seen within those Violaceae analyzed (see also Metcalfe & Chalk, 1972). Although little more is known about pith shape in the Achariaceae, it has been characterized as circular (Metcalfe & Chalk, 1972), so that the fluted stem pith of *Goupia* may be regarded as an apomorphy, and stem pith shape is mapped on Figure 1 (character states 1, 2). Metcalfe and Chalk (1972: 103) say of Violaceae that "the pith is solid in woody species, but frequently becomes hollow in herbs"; however, there is no mention of heterogeneous pith. The heterogeneous pith of *Fusispermum* is potentially an apomorphy for the genus.

As Howard (1974, 1979a, 1979b) emphasizes, the vascular anatomies of stem, petiole, and blade midrib cannot be considered in isolation. Bearing this in mind, I nevertheless particularize some observed variations to facilitate the discussion. Sinnott (1914) previously reported three-trace, three-gap nodal anatomy for the Violaceae, Passifloraceae, and Flacourtiaceae s.l., in general for plants with stipules. Such trilacunar stem nodes are found throughout the Malpighiales, with the exception of the Bonnetiaceae–Calophyllaceae clade (cf. Stevens, 2012). The eight species studied here for *Rinorea* and *Goupia* shared these trilacunar nodes, with the exception of *Fusispermum*, which had pentalacunar stem nodes. In the context of Malpighiales, these pentalacunar nodes are likely to be an apomorphy and are mapped as such (Fig. 1).

The most conspicuous gross variation for leaf midrib anatomy in the species studied here was observed for the number of adaxial to lateral bundles and the tissue configuration in the central vascular bundle of the midrib. Although *Goupia glabra* and *Fusispermum laxiflorum* had medullary plates of vascular tissue associated with the central bundle, the midrib vasculature was very different in morphology. Metcalfe and Chalk (1972) mention that in *G. glabra*, xylem was adaxially presented and phloem abaxial (1972). However, in *F. laxiflorum*, xylem entirely surrounded phloem in the latter, which is rather unusual and probably an apomorphy (Fig. 2H). *Rinorea apiculata* has an open central bundle, while the midrib vasculature in the other *Rinorea* species examined is annular.

Although this anatomical variation was not optimized on the summary tree, the structure of the petiole bundle (and associated nodal anatomy) throughout the family will require further examination. For the herbaceous *Viola*, Metcalfe and Chalk (1972) report an arcuate central vascular strand in the petiole accompanied by one or two rib strands on each side. There are possible correlations with leaf insertion. Thus, *Fusispermum laxiflorum*, *F. minutiflorum*, *F. rubrolignosum*, *Rinorea apiculata*, *R. crenata*, and *R. paniculata*, all with distichous phyllotaxy, have two pairs of rib bundles. It will be interesting to see if other species in *Rinorea* s. str. that have been reported to have such ranked or alternate leaves have similar vasculature. It is confirmed here that *R. viridifolia*, *R. dasyadena*, and *R. squamata*, which have opposite leaves and symmetric lamina bases, have only a single rib bundle on either side of the petiole. *Rinorea lindeniana*, with two rib bundles on one side and one on the other (Fig. 3E), presents an interesting

anomaly. It has distichous leaves with very strongly asymmetric lamina bases, which may account for the odd number of vascular bundles.

All 10 species of the Violaceae (*Rinorea*, *Fusispermum*) studied here had calcium oxalate crystals (as rhombic crystals and druses) in the collenchyma. Only *Goupia glabra* lacked such rhombic crystals of calcium oxalate in the collenchyma tissue.

The thickness of the leaf lamina found here for the 10 Violaceae sampled may correlate with environmental conditions (Dickison & Weitzman, 1996; Alvarenga & Lombardi, 2010). *Fusispermum laxiflorum*, with the thickest blades at 270 μm, can reach the forest canopy, being a tree up to 20 m or more, while those *Rinorea* species studied are understory species from 2 to 10 m tall. In the sampled species, the leaves of *Rinorea* ranged from 150 to 180 μm, and these trees never reached the canopy. Both *Goupia glabra* and *F. laxiflorum* had similar leaf blades with well-developed palisade tissue as sampled, and both were trees that reach the canopy. The *Rinorea* species studied have poorly organized palisade tissue, and all were collected from smaller plants. It should be noted that *R. apiculata* can grow to heights of 30 m (Hekking, 1988). Palisade tissue tends to be better developed under well-insulated conditions (e.g., Dickison & Weitzman, 1996; Alvarenga & Lombardi, 2010). Some species of *Viola* have been noted to have no clearly differentiated palisade tissue (Metcalfe & Chalk, 1972), but the genus is herbaceous and may occur in somewhat shaded conditions.

The structure of the midrib largely mirrored that of the petiole for the 11 species sampled. In *Rinorea apiculata*, with its incurved-arcuate primary petiole bundle, the midrib was fully bilayered, as in the other species of *Rinorea* s. str. examined. In *Goupia glabra* the midrib was also bilayered, evidence of the petiolar medullary plate being lost except for the presence of a few medullary fibers. In *Fusispermum laxiflorum*, *F. minutiflorum*, and *F. rubrolignosum*, the vascular tissue in the midrib consisted of two arcuate bands of fibers surrounding the vascular tissue, with two additional bands of vascular tissue in the midrib that were continuous with the elliptical central cylinder in the petiole.

The stamens of many Violaceae are notable for the abaxial nectar glands on some or all filaments and the complex, flattened connective (Arnal, 1945). Species of *Rinorea* s. str. have glands adnate to the androecial filament that can be either fused or free, and the variation is taxonomically significant.

Assuming that the Goupiaceae are sister to Violaceae, there appears to be an increasing elaboration of the androecial connective and a shift in the nectary from a structure more or less separate from other parts of the flower to its adnation to the filament in basal Violaceae, as is seen in the *Rinorea* exemplars, even though polarization of the anatomical variation was difficult to distinguish. The rather massive disc surrounding the staminal whorl in *Goupia glabra* and *Fusispermum laxiflorum* is similar to the nectaries of other members of the parietal placentation group in the Passifloraceae, Violaceae, and Salicaceae (Stevens, 2012), with the nectary lobes alternating with the filaments. For the seven species of *Rinorea* examined, the nectary was more specifically associated with the abaxial part of the filament. The enlarged, hemispherical disc of the *R. apiculata* group (*R. apiculata*, *R. crenata*, and *R. oraria*) is morphologically similar to the thickened disc of *Fusispermum* and *Goupia*, while in the other species of *Rinorea* s. str. the nectariferous tissue is more closely associated with the filament, which is the more common condition seen elsewhere in the family.

These characters—the nectary lobes alternating with or opposite to filaments, the nectary disclike, and the gland adnate to the filament—are included on the tree (see Fig. 1). However, more detailed comparative studies on the stamens/nectaries in Violaceae are likely to disclose more phylogenetically interesting information.

The anther connective is stout and rounded in *Goupia glabra* but flattened and relatively thin in all other species sampled. *Fusispermum laxiflorum, F. minutiflorum*, and *F. rubrolignosum* have a very short, erose connective ca. 80 μm long, while the longest connective occurs in *Rinorea viridifolia* (ca. 2 mm long). In the *R. apiculata* group (*R. apiculata*, *R. crenata*, and *R. oraria*), the anther thecae are visible in adaxial view, while in the other species they are concealed. The connective scales are known to vary in size and shape among other Violaceae (Engler, 1925; Arnal, 1945).

Variation in the gynoecia of the 11 species sampled is of considerable interest. The axile placentation and basal ovules of the outgroup *Goupia glabra* (see, e.g., Miers, 1862) are unique among the parietal placentation group in the Malpighiales and may be hypothesized as apomorphies, as are the 5-carpellate gynoecium and marginal styles (uncommon in Malpighiales). However, details of the stigmatic area of both *Goupia* and those Violaceae sampled may well be similar: the stigmatic area is not expanded, and there is a central depression or pore noted in all (Fig. 4E, F, H). More noteworthy in the *Rinorea apiculata* and *R. crenata* (Fig. 4G), the apex of the style flares outward a likely apomorphy.

Most Violaceae studied had straight styles (Fig. 4H), but in *Rinorea lindeniana* the style is curved at the base (Fig. 4I), and in *R. paniculata* the style is sigmoid at the base. In genera such as *Hybanthus*, *Amphirrhox* Spreng., *Paypayrola* Aubl., and *Gloeospermum* Triana & Planch., the styles are straight, consistent with the family (Steyermark et al., 2005). In *Rinoreocarpus ulei* (Melch.) Ducke and other species of *Rinorea* and *Viola*, the style has been noted as slightly curved (Engler, 1925; Arnal, 1945; Hekking, 1988). The taxonomic significance of the style curvature needs further investigation within the family.

Of the species studied, only *Goupia* and *Fusispermum* have more than a single ovule per carpel (Hekking, 1984), which is probably the plesiomorphic condition for Violaceae as a whole. Although the seven species of *Rinorea* examined consistently had only one ovule per carpel, there is considerable variation in ovule number in other Violaceae, and the phylogenetic utility of the character is not clear.

It has been suggested that the flowers of *Rinorea* (see Arnal, 1945) and *Fusispermum* (Hekking, 1988) species are actinomorphic. Recent studies of floral development in *Rinorea* demonstrate that the polysymmetry of this actinomorphy is achieved late in the ontogeny. Almost actinomorphic to very weakly zygomorphic flowers are noted in *Gloeospermum* (Steyermark et al., 2005), and flowers that are actinomorphic in early stages of development in *Leonia* Ruiz. & Pav. become zygomorphic later (Feng, 2005). Feng and Ballard (2005) suggest that the family had a most recent common ancestor with zygomorphic monosymmetric, rather than actinomorphic, flowers. However, early developing flower buds of *F. laxiflorum* and two species of *Rinorea* (i.e., *R. lindeniana* and *R. dasyadena*) have been seen to be zygomorphic with a more or less oblique orientation (Hoyos-Gómez, pers. obs.), which might suggest that zygomorphic flowers may indeed be a synapomorphy for the Violaceae.

There is interesting variation in the seed coat among the Violaceae, and studies by Corner (1976), Plisko (1992), and Melikian and Sarinov (2000) have placed this variation in phylogenetic context. When studying seed coat anatomy, ideally one should look at developmental stages so that one can follow the development of particular portions of the integuments as incorporated into the seed coat (e.g., Corner, 1976). Such developmental series could not be observed here, so the attribution of cells to the tegmen is inferred on the number of layers to the testa, the presence of a cuticle, and so forth.

The filiform seeds of *Fusispermum minutiflorum* have longitudinal ridges or wings 11 to 16 cells across, with the embryo occupying about half the length of the seed. This differed from the ovoid to ellipsoid seeds of the other taxa examined here and also differed from seeds of the Achariaceae, Passifloraceae, and the like (Malpighiales). The winged, filiform seed shape noted for species of *Fusispermum* can be considered an apomorphy and is linked to differences in seed coat anatomy.

The exotegmen of the seed wall consisted of a more or less well-developed layer of lignified cells, all cell walls of which were more or less evenly thickened in the *Fusispermum* and *Rinorea* species sampled (but not in *Goupia glabra*), and there were fine plasmodesmata evident across the cell walls (Fig. 5E, F). In *G. glabra* the testa was at least two to three cell layers across, and there was a single layer of massively cubical cells, presumably exotegmic (Fig. 5C). In the *R. apiculata* group (with *R. crenata*) the testa was three to four cell layers across, and the sclerified layer consisted of a single layer of obliquely overlapping cells (Fig. 5F). In the other five species of *Rinorea* s. str. examined, the seed coat was eight to 10 cell layers across, of which four to six layers were lignified, the cells being elongated and flat lying (Fig. 5H).

A single layer of sclerified cells in the exotegmen was found in all Violaceae studied by Corner (1976), Plisko (1992), and Melikian and Sarinov (2000), including the berry-fruited *Leonia*. But for species like *Rinorea anguifera* Kuntze (Plisko, 1992), four to five layers of sclerified cells were observed. In this study, *R. dasyadena*, *R. lindeniana*, and *R. squamata* have similar, multilayered sclerified tegmic tissue. Note that Melikian and Sarinov (2000) show the seed coat of *Goupia glabra* as having mesotestal sclereids. However, although the material examined here was limited, there was clearly more than one layer of cells above the sclerified layer, suggesting that the sclerified layer is exotegmic. This layer was also definitely longitudinally ridged, another feature not noted by Melikian and Sarinov (2000), who placed the Goupiaceae near Celastraceae, which would commonly have a lignified mesotesta.

Some kind of exotegmen made up of more or less fibrous cells is common in the Malpighiales (e.g., Corner, 1976; Stevens, 2012). Details of cell elongation and the degree, and plane, of the cellular flattening have varied and need to be integrated with phylogeny across the clade. However, despite our still limited knowledge of seed coats in the Violaceae, the four or five layers of fibrous cells found in the species

of *Rinorea* s. str. studied, as a provisional finding, can be considered unique.

The embryo in *Goupia glabra*, *Fusispermum minutiflorum* (Fig. 5D), and the *Rinorea apiculata* clade was straight, with flat cotyledons, a condition considered to be normal for the family (e.g., Plisko, 1992). However, in the other examined species of *Rinorea* s. str., the cotyledons exhibited complex folds. Vogel (1980) noted that *Rinorea* species had seedling type I (*Macaranga* Thouars–type), where cotyledons in most cases were more or less folded within the seed. Further investigation of embryo morphology would repay the effort with characters of phylogenetic significance.

Examination of the androecium and nectary in those Violaceae and Goupiaceae examined relates the distinctive androecium so common in Violaceae with more conventional structures found in other members of the parietal placentation group within the Malpighiales. Although many features of the gynoecium of *Goupia* are quite different from those of the parietal classification group, the two families had not been previously associated, not until support for the sister relationship of the Goupiaceae to the Violaceae was corroborated by molecular evidence. Otherwise, in the morphology of the stigma and androecial features, as well as in basic anatomy, the Violaceae and Goupiaceae agree quite well. Without such detailed anatomical and morphological knowledge we cannot hope to understand evolution and diversification, not only of Violaceae but of Malpighiales as a whole, such an important component of the small trees that are so abundant in tropical rainforests (Davis et al., 2005).

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